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Lipid-induced pramlintide release from genetically modified cells transiently reduces weight gain in rats

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1 ZUSAMMENFASSUNG

Amylin ist an der Kontrolle der Energiehomöostase beteiligt und verstärkt das Leptinsignal im mediobasalen Hypothalamus und möglicherweise im kaudalen Rhombencephalon. Der Amylin-Agonist Pramlintide wird zusammen mit Insulin als Therapie bei Typ-1 und Typ-2 Diabetes eingesetzt, um die postprandiale Glukosekonzentration im Blut besser kontrollieren zu können. Pramlintide reduziert auch das Körpergewicht und die Energieaufnahme. In dieser Studie wurde ein neues Verfahren angewendet, um Pramlintide kontrolliert zu verabreichen. HT-1080-Zellen wurden mit dem sogenannten lipid-sensing receptor (LSR) Plasmid (pKR135) und dem LSR-kontrollierten Pramlintide Plasmid (pKR146) transfektiert. Die Zellen wurden in Algin-poly-(L-Lysine)-Algin Mikrokapseln eingekapselt und intraperitoneal männlichen Sprague-Dawley Ratten injiziert, die mit high fat diet (60% Fett; Research Diets) gefüttert wurden. 20 x 10^6 Zellen (pKR135/pKR146 HT-1080 Zellen) wurden pro Ratte injiziert; der Kontrollgruppe wurden pKR135 HT-1080 Zellen implantiert. Ratten, die mit Pramlintide-sezernierenden Zellen injiziert wurden, zeigten eine signifikant reduzierte Futteraufnahme am Tag 1, und im Vergleich zu den Kontrollratten eine reduzierte kumulative Körpergewichtszunahme von Tag 2 bis Tag 5. Pramlintide verringerte auch die Futteraufnahme während einer Testmahlzeit um 26% ($P<0.01$) am Tag 3 nach Applikation. Dies war mit einer Zunahme der Plasma Pramlintidekonzentration assoziiert. Die Ratten mit den Pramlintide-sezernierenden Zellen, zeigten 4 Stunden nach der Injektion von Leptin am Tag 5 und 7 eine um 33 % ($P<0.05$) verstärkte Leptinwirkung auf die Futteraufnahme. Diese Studie zeigt die erfolgreiche, jedoch relative kurzwirksame Wirkung eines autonomen Systems bei Ratten in den die Pramlintide-Produktion und –Sekretion durch eine Hochfettdiät kontrolliert wurde.

2 SUMMARY

Amylin is an important control of energy homeostasis and it enhances leptin signaling in the mediobasal hypothalamus and possibly the caudal hindbrain. The amylin analogue pramlintide has been used clinically as an adjunct to insulin therapy in type 1 and 2 diabetes to better control postprandial glucose level and has been previously shown to reduce body weight and energy intake. Here, a new approach was used to deliver pramlintide. HT-1080 cells were co-transfected with the lipid-sensing receptor (LSR) plasmid (pKR135) and the LSR-controlled pramlintide plasmid (pKR146). These cells were microencapsulated in alginate-poly-(L-lysine)-alginate beads and injected intraperitoneally in male Sprague-Dawley rats maintained on high fat diet (60% fat; Research Diets). 20 x 10^6 cells (pKR135/pKR146-engineered HT-1080 cells) were injected per rat; the control group was implanted with pKR135-engineered HT-1080 cells. Rats injected with the pramlintide secreting cells had significantly lower food intake at day 1, and lower cumulative body weight gain from day 2 to day 5 as compared to control rats. Pramlintide also reduced food intake by 26% ($P<0.01$) during a meal test on day 3; this effect was associated with an increase in plasma pramlintide levels. Leptin's effect on 4h-food intake was amplified by 33% ($P<0.05$) in rats injected with pramlintide beads tested 5 and 7 days after implantation. The current study demonstrated the successful but transient effect of an autonomous genetic system providing fatty acid-induced production and secretion of pramlintide in rats put on high fat diet.

3 INTRODUCTION

Amylin is an important control of energy homeostasis (Young and Denaro 1998, Young 2005, Lutz 2010, Lutz 2012). It is co-secreted with insulin in response to nutrients by the pancreatic β-cells which are the major source of circulating plasma amylin (Ogawa, Harris et al. 1990, Young and Denaro 1998). Amylin secretion undergoes meal-associated fluctuations like a postprandial increase that is responsible for amylin's satiating effect and its action on energy homeostasis (Young and Denaro 1998, Young 2005, Lutz 2010), but amylin also fulfills the criteria of long-term adiposity signals (Lutz 2010, Lutz 2012). Its short-term effects are to reduce food intake by inducing satiation and by reducing meal size (Lutz, Del Prete et al. 1994, Morley, Flood et al. 1994, Lutz, Geary et al. 1995), to inhibit gastric emptying (Young, Gedulin et al. 1995, Young, Gedulin et al. 1996, Young 2005), and to inhibit postprandial glucagon secretion (Gedulin, Rink et al. 1997, Young 2005, Gedulin, Jodka et al. 2006). As an adiposity signal, like leptin or insulin, its concentration follows levels of body fat and it contributes to body weight regulation (Pieber, Roitelman et al. 1994, Rushing, Hagan et al. 2000, Roth, Hughes et al. 2006, Mack, Wilson et al. 2007, Boyle and Lutz 2011). Amylin's primary site of action is located in the area postrema (AP) (Lutz, Senn et al. 1998, Potes and Lutz 2010, Braegger, Asarian et al. 2014). It binds to membrane-bound receptors that are heterodimers of the calcitonin receptors and receptor activity-modifying proteins (RAMPs), both present in the AP (Christopoulos, Perry et al. 1999, Muff, Buhlmann et al. 1999, Morfis, Tilakaratne et al. 2008, Liberini, Boyle et al. 2016). Amylin's binding in the AP induces neuronal activation represented by expression of the marker c-Fos (Riediger, Zuend et al. 2004), the acute phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Morfis, Tilakaratne et al. 2008, Potes, Boyle et al. 2012) and the formation of the second messenger cyclic guanosine monophosphate (cGMP) (Riediger, Schmid et al. 2001). Activation of AP neurons through amylin is transmitted on a neuroaxis that includes several hypothalamic nuclei such as the ventromedial hypothalamus (VMH) (Mollet, Meier et al. 2003, Potes, Lutz et al. 2010).

Amylin is also known to enhance leptin signaling in the mediobasal hypothalamus, resulting in a synergistic effect on eating, body weight and body adiposity (Trevaskis, Lei et al. 2010, Trevaskis, Parkes et al. 2010, Turek, Trevaskis et al. 2010, Le Foll, Johnson et al. 2015, Dunn-Meynell, Le Foll et al. 2016). Leptin is produced in the white adipose tissue and acts via the systemic circulation on arcuate nucleus (ARC) neurons, located in the mediobasal hypothalamus. Leptin binding activates amongst other anorexigenic proopiomelanocortin

(POMC) neurons. Leptin activates several signaling pathways, e.g. the janus kinase 2/Signal Transducer and Activator of Transcription 3 (JAK-STAT) signaling is involved in leptin's effect on food intake (Gao, Wolfgang et al. 2004) whereas ERK1/2 signaling plays an important role in the regulation of energy homeostasis (Rahmouni, Sigmund et al. 2009). Phosphorylated STAT3 (pSTAT3) facilitates the leptin-mediated transcription of POMC (Bouret, Bates et al. 2012, Kwon, Kim et al. 2016). Amylin increases leptin-induced pSTAT3 in the ARC (Turek, Trevaskis et al. 2010) and restores the leptin-induced pSTAT3 in the VMH of obese rats (Roth, Roland et al. 2008, Trevaskis, Coffey et al. 2008). Co-treatment with amylin and leptin also increases leptin binding in the ARC (Lutz 2010, Turek, Trevaskis et al. 2010).

In recent years, amylin agonists have been developed to mimic amylin activity. Pramlintide is a synthetic analog of human amylin that differs from it in 3 amino acids and exhibits similar pharmacology to rat amylin (Young 1996, Weyer, Maggs et al. 2001, Gingell, Burns et al. 2014). Pramlintide has been used clinically as an adjunct to insulin therapy in type 1 and 2 diabetes to better control postprandial glucose level, but pramlintide also reduced body weight and energy intake in diabetics and non-diabetics (Younk, Mikeladze et al. 2011).

Rössger et al. (Rossger, Charpin-El-Hamri et al. 2013) recently described a novel method to deliver pramlintide in a diet-specific manner in mice. They implanted a designed closed-loop synthetic gene circuit that monitors blood fatty acid levels and produces pramlintide in response to elevated fatty acids. This led to a reduced food intake and body weight and decreased blood fat levels compared to respective controls. The aim of our study was to test the usefulness of this new approach in rats and for a longer time period; we injected the same lipid sensing cells that are activated by increased fatty acid levels. Further, we tested whether the synergistic effects of leptin and amylin receptor activation is also present under these conditions.

4 MATERIAL AND METHODS

4.1 In vitro experiments

4.1.1 Plasmids

Three different engineered plasmids were used in this study (Fig. 1) (Rossger, Charpin-El-Hamri et al. 2013). The lipid-sensing receptor (LSR) plasmid (pKR135) is a transcription factor that results from a fusion of the ligand-binding domain of the human nuclear lipid receptor Peroxisome Proliferator-Activated Receptor-α (PPARα), which possesses a receptor for fatty acids, and the bacterial DNA-binding repressor TtgR that binds the chimeric promoter P_{TtgR1}. The LSR-controlled serum alkaline phosphatase (SEAP) plasmid (pMG10) was used as a control plasmid and contains the P_{TtgR1} domain. Following co-transfection, pKR135 binds to pMG10 and, in the absence of fatty acids, LSR associates with an endogenous inhibitory complex to repress transgene expression. SEAP secretion is triggered by fatty acids and an endogenous activation complex, which both bind to the PPARα domain of pKR135. The LSR-controlled pramlintide plasmid (pKR146) also contains the P_{TtgR1} and secretes pramlintide following specific binding with the pKR135 plasmid and the presence of fatty acids.

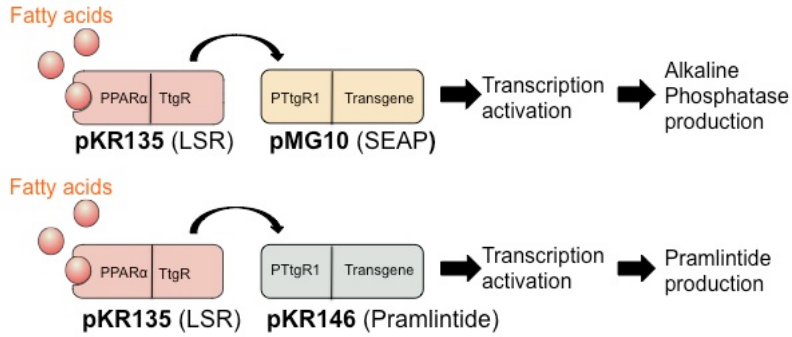

Figure 1: Lipid-sensing receptor (LSR) plasmid (pKR135) possesses the receptor for fatty acids PPARα and the repressor TtgR. LSR-controlled alkaline phosphatase (SEAP) plasmid (pMG10) and LSR-controlled pramlintide plasmid (pKR146) both possess the promoter P_{TtgR1}. Presence of fatty acids activates SEAP and pramlintide transcription and production. Plasmids engineered by Rossger et al. (Rossger, Charpin-El-Hamri et al. 2013)

One Shot® TOP10 Chemically Competent E.coli (Cat.no.C404003, LifeTechnologies, Carlsbad, USA) were transformed with one plasmid at the time. The transformed cells were spread on LB medium agar plates, selected with ampicillin (100 μg/ml) and incubated at 37°C

overnight. Two isolated colonies per plate were then selected and used for the growing of two successive colonies in liquid LB medium + ampicillin and placed in a shaking incubator at 37°C overnight. The plasmid DNA was isolated using the QIAGEN® Plasmid Purification Maxi Kit (Cat. No.12162, QIAGEN AG, Hombrechtikon, Switzerland). The quantity and quality of each plasmid was verified using a nanodrop (Thermo Fisher AG, Reinach, Switzerland).

4.1.2 Cell culture and transfection

Human fibrosarcoma cells HT-1080 (HT1080, ATCC® CCL-121™, Manassas, USA) were cultivated in a growth media (EMEM, ATCC® 30-2003™, Manassas, USA) supplemented with 1% penicillin/streptomycin solution and 10% fetal bovine serum solution. Cultures were kept at 37°C and 5% CO2 environment. Twelve hours prior to transfection, 50'000 cells per well were seeded in a 24-well plate containing 0.5ml of growth media per well without antibiotics. Cells were co-transfected and incubated for 6h with the transfection solution containing per well 1μl of transfection reagent Lipofectamine® 2000 (Cat.no.11668019, LifeTechnologies, Carlsbad, USA), 49μl of Reduced Serum Medium OPTI-MEM® (Cat.no.31985062, LifeTechnologies, Carlsbad, USA) and different plasmid combinations (50ng pKR135 + 250ng pMG10; 125ng pKR135 + 250ng pMG10; 250ng pKR135 + 250ng pMG10; 250ng pKR135 + 500ng pMG10; 250ng pKR135 + 250ng pKR146; 250ng pKR135 + 500ng pKR146). For control, cells were treated identically except for the transfection of plasmids (non-transfected cells). Cells were then kept in a complete growth media for 12 hours after which 100μl cell culture supernatant was sampled per well and 100μM linoleic acid was added to certain wells (treated wells). This process was repeated at 24h and 48h post-linoleic acid treatment. Cells from transfected non-treated wells were exposed to the growth media only. Cell culture supernatant samples were stored at -20°C until further analysis of SEAP concentration (pKR135/pMG10-cotransfection) and pramlintide concentration (pKR135/pKR146-cotransfection).

4.1.3 Transfection for the in vivo animal experiment

Co-transfection of HT-1080 cells for the in vivo animal experiment was processed similar to previous transfections, only adjusted to the increased number of transfected cells. Thus 10^7 cells plated the day before were co-transfected with the transfection solution containing per plate 225μg pKR135 plasmid and 500μg pKR146 plasmid; for control, plates were

transfected with 225μg of pKR135 plasmid. Cells were incubated for 6h as above, trypsinated and the pellet was reconstituted in a MOPS/Alginate (1.8% NA Alginate, Büchi, Flawil, Switzerland) solution with a 1:5 ratio. Cells were then microencapsulated in coherent alginate-poly-(L-lysine)-alginate beads (400 mm; 200 cells per bead) using the Encapsulator B-395 Pro (Büchi) set to the following parameters: 200 mm nozzle with a vibration frequency of 1,020 Hz and 950V for bead dispersion, 20 ml syringe operated at a flow rate of 4.5 ml/min.

4.2 In vivo experiment

4.2.1 Animal experiment

Twelve single-housed male Sprague-Dawley (Janvier Labs, France) rats were maintained in a 12:12-h dark-light cycle (lights off at 10:00h). Rats were fed high fat diet (DIO Formulas D12492, 60% kcal, Research Diets, New Brunswick, USA) throughout the entire study with free access to water and food unless indicated otherwise. Rats were acclimated to housing conditions 1 week before the study. Body weight and food intake were measured daily. All animal experiments were approved by the Veterinary Office of the Canton of Zürich, Switzerland.

On day 0, rats were injected intraperitoneally with 4ml serum free media containing $20x10^6$ encapsulated cells. The control group (n=6) was implanted with pKR135-engineered HT-1080 and the pramlintide group (n=6) with pKR135/pKR146-engineered HT-1080 cells. Blood was collected 1 day prior to the beads injection (baseline), on day 3 directly before and after the meal test (0h and 4h) and at sacrifice on day 13. Serum was isolated and stored at -20°C for further analysis.

4.2.2 Food intake

A meal test was performed at day 3 after beads implantation. Food was removed 4h before dark onset and food intake was then measured for the first 4h after dark onset. A leptin induced anorexia test was performed at day 5 and 7 using a crossover paradigm. Food was removed 2h before dark onset, after which rats were injected intraperitoneally either with leptin (IP, 5mg/kg) or with PBS buffer. Food intake was then measured at 4h and 24h after leptin injection.

4.2.3 Perfusion and brain processing

Rats were sacrificed 13 days after beads implantation. All rats were injected with leptin (IP; 5mg/kg) at dark onset after a 2h fast. Rats were then deeply anesthetized with sodium pentobarbital (IP, 50mg/kg, Kantonsapotheke Zürich, Switzerland) 45 minutes later. Blood was collected transcardially. A transcardiac perfusion with ice-cold 0.9% NaCl for 1.5 minutes followed by 0.1M phosphate buffered saline (PBS)-4% paraformaldehyde (PFA) for 2.5 minutes was performed. Brains were removed, postfixed in PFA 4% at 4 °C overnight and cryoprotected in 20% sucrose/PBS at 4°C overnight under constant agitation. They were then frozen for 3 minutes in hexane (-20°C) and stored at -80°C until sectioning. Brains were sectioned coronally on a cryostat (model CM3050S; Leica, Nussloch, Germany) and four series of 30-μm sections were cut through the midpoint of ARC, VMH and dorsomedial nucleus (DMH) pars compacta of the hypothalamus. The sections were stored in cryoprotectant at -20°C until immunohistochemistry.

4.3 Cell culture supernatant and blood analysis

SEAP concentration was assessed with the chemiluminescent SEAP reporter gene assay (Roche Diagnostics GmbH, Rotkreuz, Switzerland) on a microplate fluorescence reader (FLx800™, BioTek™). Pramlintide and leptin levels were measured using the human amylin Elisa kit (quantification of endogenous amylin and pramlintide) and rat leptin Elisa kit (EZHA-52K, EZRL-83K, EMD Millipore Corporation, Missouri, USA), respectively, on a microplate photometer (Multiskan™ RC, Thermo/Labsystems). Insulin levels were assessed using the rat insulin Elisa kit (Cat.no.10-1250-01, Mercodia, Uppsala, Sweden). Plasma samples for the analysis of glucose, HDL cholesterol and triglycerides levels were sent to the Zurich Integrative Rodent Physiology and analysed with the UniCel® DxC 800 Synchron® Clinical System (Beckman Coulter, Nyon, Switzerland). Cytokine (IFγ, Il-10, Il-1β, Il-6, TNF-α) levels at sacrifice were determined using the proinflammatory panel 1 (mouse) kit (MSD® Multi-spot Assay System, Rockville, USA) and read on a Mesocale MSD instrument.

4.4 pERK1/2 and POMC immunohistochemistry

Three brain sections per rat containing the ARC/VMH of the hypothalamus were selected and were incubated for 20 min in 0.02M potassium phosphate buffered saline (KPBS) containing 0.5% NaOH and 0.5% H_2O_2, followed by two successive incubations in 0.3% glycine and 0.03% sodium natriumlaurylsulfat (SDS). Sections were rinsed in KPBS for 5x5 min after

each incubation. After an 1h incubation in blocking solution containing 4% normal goat serum (NGS), 0.4% Triton and 1% Bovine Serum Albumin (BSA), the sections were incubated for 48h at 4°C in KPBS solution containing 1% NGS, 0.4% triton, 1% BSA and phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) antibody diluted 1:1000 (Cat No 9101, Cell Signaling Technology, Danvers, USA). On day 3, sections were rinsed in KPBS for 8x5min and incubated for 2h at room temperature in KPBS containing 4% NGS, 0.4% triton and goat anti-rabbit IgG (H+L) secondary antibody (alexa fluor® 555 conjugate, Cat No A-21429, ThermoFisher, Waltham, USA) diluted 1:400. Sections were rinsed again in KPBS, incubated in blocking solution for 1h (4% Normal Donkey Serum (NDS), 0.4% Triton and 1% BSA) and incubated for 48h at 4°C in KPBS containing 1% NDS, 0.4% triton, 1% BSA and POMC Precursor (27-52)(Porcine) Antibody diluted 1:1000 (Cat No H-029-30, Phoenix Pharmaceuticals Inc., Burlingane, USA). On day 5, sections were rinsed in KPBS, incubated for 2h at room temperature in KPBS containing 1% NDS, 0.4% triton, 1% BSA and donkey anti-rabbit IgG (H+L) secondary antibody (alexa fluor® 488 conjugate, Cat No A21206, ThermoFisher, Waltham, USA), diluted 1:250. Sections were rinsed one last time and mounted on gel-coated slides and coversliped with Citifluor (glycerol/PBS 1:1; Citifluor Products).

4.5 Microscopy

Brain sections were analysed at 10 and 40-fold magnification using a microscope equipped with a digital camera (Axio Imager 2, Zeiss, Germany). Images were processed with the microscope software AxioVision (Zeiss, Germany). The number of pERK1/2 and POMC single-immunoreactive neurons and pERK/POMC double-immunoreactive neurons was quantified in the ARC and the VMH bilaterally. Counting of single-immunoreactive cells in each region was conducted with the image processing program ImageJ (National Institute of Health, Bethesda, MD). Counting of double immunoreactive cells was conducted manually. All counting were conducted by an experimenter blinded to the treatment.

4.6 Statistics

In vitro data comparing more than two groups were analysed using two-way ANOVA with post hoc comparisons. In vivo data comparing two groups were analysed using Student *t*-test. Statistical significance was assumed for $P<0.05$. Graphs were generated using Prism 5 for Mac (GraphPad Software, San Diego, USA). All data are presented as mean ± SEM.

5 RESULTS

5.1 Fatty acid-induced SEAP and pramlintide production in vitro

5.1.1 SEAP production in vitro

SEAP concentration of non-transfected cells (n=4) and co-transfected cells (pKR135/pMG10) (n=4) was assessed before linoleic acid treatment (0h) and at 24h and 48h post-treatment (Table 1; Fig. 2A). Different concentrations and combinations of the two plasmids, pKR135 and pMG10 (50ng pKR135 + 250ng pMG10; 125ng pKR135 + 250ng pMG10; 250ng pKR135 + 250ng pMG10; 250ng pKR135 + 500ng pMG10), were assessed in order to determine the best proportion to use. Prior to linoleic acid treatment, all wells had a SEAP concentration inferior to 200mU/L. Non-transfected cells and transfected cells that were not exposed to LA (non-treated wells) never exceeded this concentration. At 24h and 48h, SEAP concentration rose in linoleic acid-treated wells. The effect was dose-dependent, with the highest SEAP concentration reached by the highest plasmid concentration combination (250ng pKR135 + 500ng pMG10; 1090.4±252.4mU/L). At 48h, all treated wells except the 125ng pKR135 + 250ng pMG10 wells had a significant increase in SEAP concentration when compared with one or both non-treated wells.

5.1.2 Pramlintide production in vitro

Pramlintide concentration of non-transfected cells (n=4) and co-transfected cells (pKR135/pKR146) (n=4) was assessed before linoleic acid treatment (0h) and at 24h and 48h post-treatment (Table 1; Fig. 2B). The initially measured pramlintide concentration was below 2.3pM in all wells. The pramlintide concentration in non-transfected cells and transfected cells that were not exposed to LA (non-treated wells) did not differ over time. At 48h, 250ng pKR135 + 250ng pKR146 and 250ng pKR135 + 500ng pKR146 linoleic acid-treated wells had a pramlintide concentration of 6.22±0.36pM and 5.68±0.29pM respectively which was significantly higher (+129 and +100%, resp.; $P<0.05$) than the pramlintide concentration of the non-treated wells with similar plasmid concentration combination. Based on this result, we opted to use the combination of 250ng pKR135 and 500ng pKR146 for the in vivo experiment.

Table 1

	0h	24h	48h
Alkaline phosphatase concentration (mU/L)			
Non-transfected cells	36,53 ± 6,39	36,53 ± 12,23	55,69 ± 18,06
50ng pKR135 + 250ng pMG10	100,4 ± 16,07	125,95 ± 31,93	100,4 ± 21,82
50ng pKR135 + 250ng pMG10 + 100µM LA	119,56 ± 30,41	317,55 ± 35,18	406,97 ± 53,56
125ng pKR135 + 250ng pMG10	39,81 ± 43,47	81,24 ± 22,86	170,66 ± 22,12
125ng pKR135 + 250ng pMG10 + 100µM LA	81,24 ± 18,06	221,75 ± 42,37	292,01 ± 31,93
250ng pKR135 + 250ng pMG10 + 100µM LA	48,22 ± 27,73	215,94 ± 94,6	383,65 ± 161,37
250ng pKR135 + 500ng pMG10 + 100µM LA	164,27 ± 16,07	681,61 ± 99,22	1090,36 ± 252,37
Pramlintide concentration (pM)			
Non-transfected cells	2,04 ± 0,14	2,72 ± 0,37	2,76 ± 0,38
250ng pKR135 + 250ng pKR146	1,85 ± 0,41	2,81 ± 0,4	2,72 ± 0,16
250ng pKR135 + 250ng pKR146 + 100µM LA	2,06 ± 0,23	4,24 ± 0,14	6,22 ± 0,36
250ng pKR135 + 500ng pKR146	2,23 ± 0,32	2,72 ± 0,06	2,84 ± 0,07
250ng pKR135 + 500ng pKR146 + 100µM LA	1,58 ± 0,18	3,14 ± 0,1	5,68 ± 0,29

Table 1: Alkaline phosphatase (SEAP) concentration (mU/L) and pramlintide concentration (pM) of cell culture supernatant before (0h) linoleic acid treatment of certain wells (100µM LA), 24h and 48h after fatty acid treatment. Wells were transfected with different plasmid concentration combination or not transfected (non-transfected cells). Lipid-sensing receptor (LSR) plasmid (pKR135) possesses the receptor for fatty acids PPARα and the repressor TtgR. LSR-controlled alkaline phosphatase (SEAP) plasmid (pMG10) and LSR-controlled pramlintide plasmid (pKR146) possess the promoter P_{TtgR1}. Presence of fatty acids activates SEAP and pramlintide transcription and production. Plasmids engineered by Rossger et al. (Rossger, Charpin-El-Hamri et al. 2013). Values are mean ± SEM.

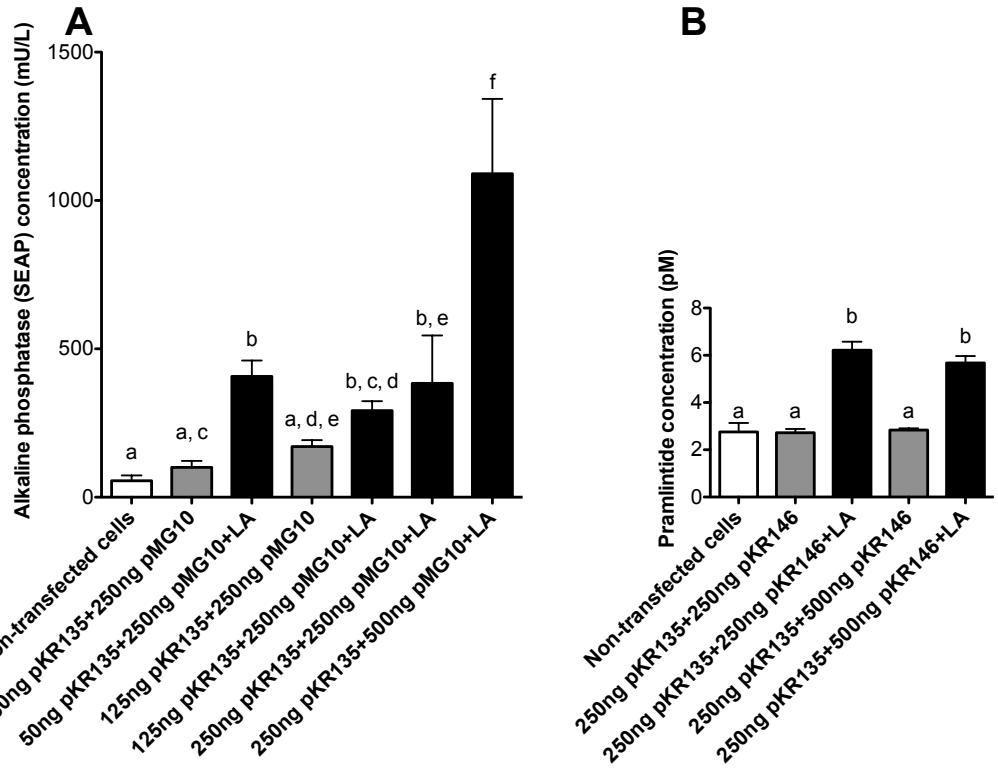

Figure 2: (**A**) Linoleic acid (LA)-induced SEAP production in HT-1080 cells at 48h. Non-transfected cells were incubated with transfection solution without plasmids, cells from non-treated wells were incubated with transfection solution and 50ng pKR135 + 250ng pMG10 or 125ng pKR135 + 250ng pMG10, and cells from treated wells were transfected with 50ng pKR135 + 250ng pMG10, 125ng pKR135 + 250ng pMG10, 250ng pKR135 + 250ng pMG10 or 250ng pKR135 + 500ng pMG10 and treated daily with 100µM linoleic acid. (**B**) Linoleic acid-induced pramlintide production in HT-1080 cells at 48h. Non-transfected cells were incubated with transfection solution without plasmids, cells from non-treated wells were incubated with transfection solution and 250ng pKR135 + 250ng pKR146 or 250ng pKR135 + 500ng pKR146, and cells from treated wells were transfected with 250ng pKR135 + 250ng pKR146 or 250ng pKR135 + 500ng pKR146 and treated daily with 100µM linoleic acid. Values are mean ± SEM. Letters indicate differences ($P<0.05$) between the groups using two-way ANOVA with Bonferroni multiple comparisons, n= 4/group.

5.2 In vivo effect of fatty acid-induced pramlintide production

5.2.1 Body weight

Initial body weight, final body weight and total body weight gain showed no significant differences between the pramlintide group and controls (Table 2). Cumulative body weight gain of rats injected with pramlintide was significantly lower from day 2 to day 5 (-12 to -

25%; P<0.05) when compared to controls and then remained similar to controls until the end of the study (Fig. 3A).

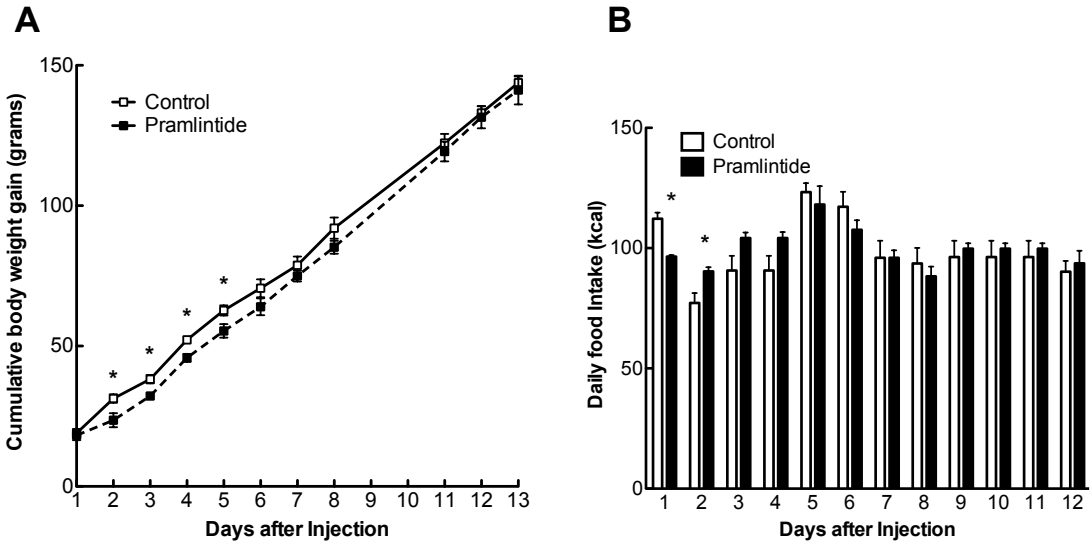

Figure 3: Cumulative body weight gain (**A**) and daily food intake (**B**) in male Sprague-Dawley rats injected at day 0 with control or LSR-pramlintide alginate beads (n=6/ group) and fed HF 60% for 12 days. Data are mean ± SEM. *P<0.05 or less using two-way ANOVA (A) and t-test (B).

5.2.2 Food intake

Rats injected with the pramlintide secreting cells had significantly lower food intake at day 1 (-14%; P<0.01) and significantly higher food intake at day 2 (+17%; P<0.05) when compared to control injected rats (Fig. 3B). Daily food intake then remained similar between both groups until the end of the study. As a consequence, final feed efficiency (BWG(g)/FI(kcal)x1000) was similar in both groups (Table 2).

Pramlintide reduced food intake by 26% (Controls = 48.6±1.4kcal Pramlintide = 36.0±3.2kcal; P<0.01) during the 4h meal test (Fig. 4A). Leptin induced anorexia was then assessed in a cross-over design on days 5 and 7 after beads implantation. Leptin's action on food intake was amplified at 4h by 34% (Controls = 94.0±8.1kcal Pramlintide = 61.7±10.2kcal; P<0.05) in rats injected with pramlintide beads (Fig. 4B). The effect of leptin on food intake at 24h was similar between both groups.

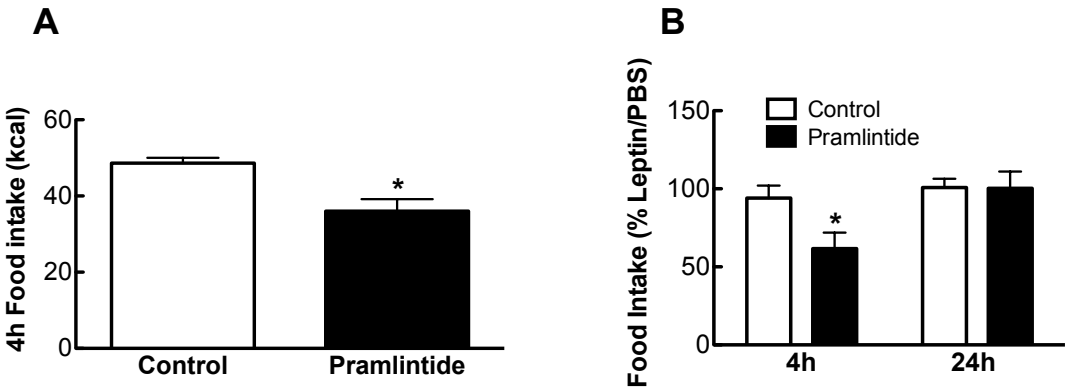

Figure 4: Food intake in kcal after (**A**) a 4h meal test on day 3 and (**B**) leptin-induced anorexia at 4h and 24h tested in a cross-over design on day 5 and 7 post-injection in male Sprague-Dawley rats injected at day 0 with control or LSR-pramlintide alginate beads (n=6/ group) and fed HF 60%. Data are mean ± SEM. *P<0.05 or less using t-test. In Figure B, 100% refers to the food intake of PBS injected rats.

5.3 Blood analysis

5.3.1 Amylin

The Elisa kit does not distinguish between endogenous amylin and pramlintide, hence the results are expressed as amylin concentration. Plasma amylin concentration before beads injection and at sacrifice showed no significant difference between the control and pramlintide group (Table 2 and Fig. 5A), but the concentration in the pramlintide group was significantly higher at sacrifice (+36%, P<0.05) compared to its baseline. This effect presumably was due to the pramlintide production by the cells. During the 4h meal test, both control and pramlintide groups showed a significant increase in plasma amylin concentration by +79% and +119%, respectively (Fig. 5A, P<0.05). The meal induced increase was stronger (+25%) in the pramlintide group but the difference to the control group was not significant (P=0.30).

5.3.2 Leptin

Blood leptin concentration was assessed before and after the meal test (Fig. 5B). Rats injected with pramlintide (0h = 6.4±0.8ng/ml, 4h = 10.9±1.3ng/ml) showed a 62% (P<0.05) increase at 4h when compared to the control group (0h = 5.5±1.1ng/ml, 4h = 6.7±0.3ng/ml) and the

significant increase within the pramlintide group between 0h and 4h (+71%; P<0.05) was not observed in the control group.

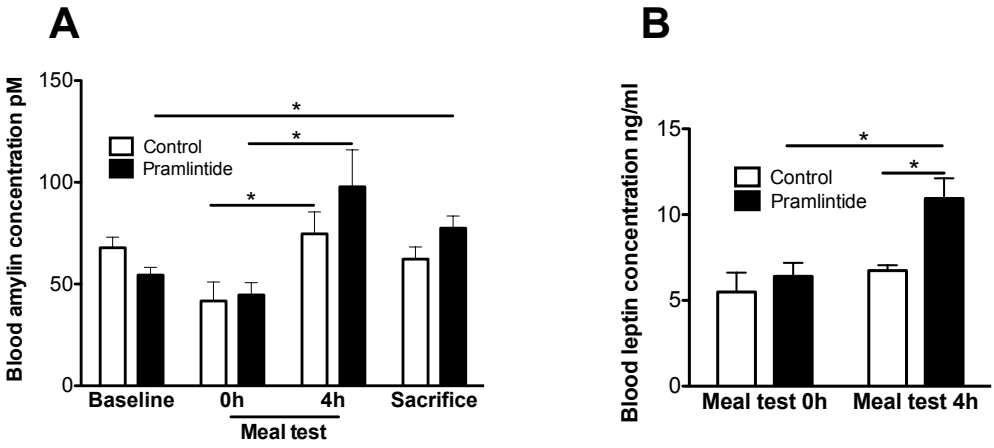

Figure 5: Blood concentration of (**A**) amylin before beads injection (Baseline), before (0h) and after (4h) meal test and at sacrifice, and of (**B**) leptin before (0h) and after (4h) meal test in male Sprague-Dawley rats injected at day 0 with control or LSR-pramlintide alginate beads (n=6/ group) and fed HF 60%. Data are mean ± SEM. *P<0.05 or less using t-test. The amylin assay does not distinguish between endogenous amylin and pramlintide.

5.3.3 Insulin and other metabolites

Insulin, glucose HDL cholesterol and triglyceride concentrations were similar between the two groups at sacrifice (Table 2).

Rats injected with pramlintide presented an 81% decrease in plasma Il-10 concentration as compared to control rats (P<0.05; Table 2). Interferon-γ, Il-1β, Il-6 and TNF-α- concentrations were similar between the control and pramlintide groups (Table 1).

Table 2

	Control	**Pramlintide**
Initial body weight (g)	274.0 ± 5.0	275.3 ± 5.5
Final body weight (g)	411.5 ± 4.3	416.5 ± 9.0
Total body weight gain on HF60% (g)	143.8 ± 2.2	141.2 ± 5.1
Total cumulative food intake on HF60% (kcal)	1184.6 ± 28.7	1187.1 ± 32.1

Feed Efficiency on chow BWG(g)/FI(kcal)x1000		119.3	± 1.7	118.2	±	3.1
Pramlintide (pM)	Baseline	67.8	± 5.2	57.01	±	5.2
	Sacrifice	62.3	± 6.0	77.5	±	6.0
Insulin (ng/ml)		5.3	± 0.3	5.5	±	0.3
Glucose (mg/dl)		186.8	± 2.9	186.0	±	3.4
HDL cholesterol (mg/dl)		36.0	± 2.1	36.6	±	3.3
Triglycerides (mg/dl)		72.7	± 19.1	95.2	±	14.8
IFγ (pg/ml)		0.02	± 0.01	0.01	±	0.004
Il-10 (pg/ml)*		0.32	± 0.09	0.06	±	0.04
Il-1β (pg/ml)		0.04	± 0.02	0.02	±	0.01
Il-6 (pg/ml)		0.96	± 0.03	0.66	±	0.12
TNF-α (pg/ml)		10.54	± 2.41	16.01	±	8.16

Table 2: Morphometric and biochemical data for male SD rats injected with control beads (n=6) or LSR-pramlintide beads (n=6) IP ($20x10^6$ cells). Rats were maintained on 60% fat diet for 13 days. Data are mean ± SEM. *P<0.05 using Student t-test.

5.4 Leptin-induced pERK and POMC expression in ARC and VMH neurons

Leptin was injected 45 minutes prior to sacrifice in order to analyse leptin-induced ERK phosphorylation and POMC expression in the ARC and VMH and to test pramlintide's effect on this signaling cascade. Leptin induced-pERK signaling in the ARC and VMH did not differ between the control group and the pramlintide group (Fig. 6A), and pramlintide had no effect on the number of POMC neurons in the ARC (Fig. 6B). Rats injected with pramlintide did not express a greater number of pERK and pERK+POMC positive neurons (Fig. 6A, C-I) in the ARC. pERK activated cells in the VMH increased markedly but not significantly in the pramlintide group (+106%; P>0.2) as compared to the controls. However the number of pERK activated neurons was approx. 10 times less in the VMH than in the ARC.

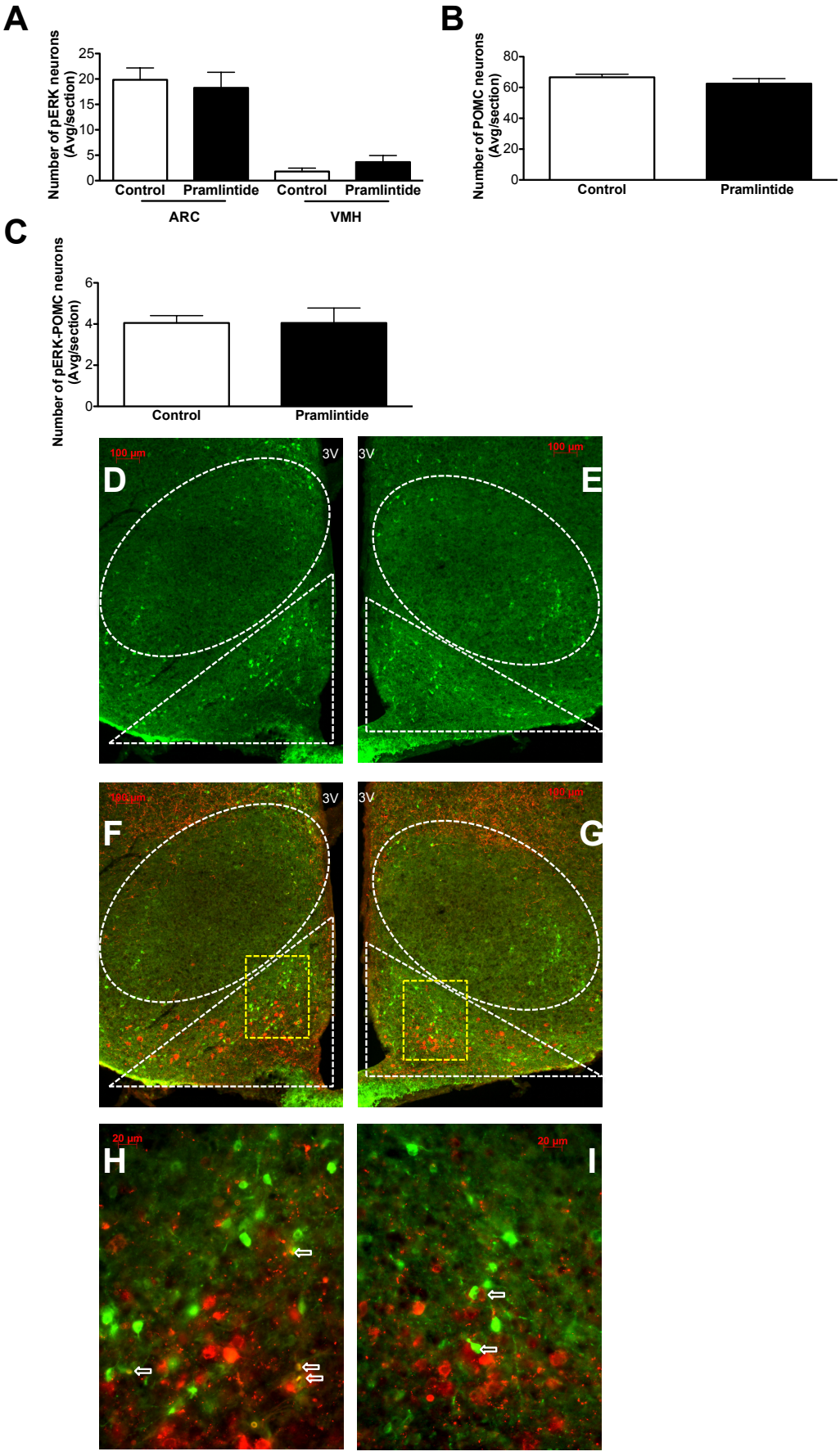
A
Number of pERK neurons (Avg/section)
25
20
15
10
5
0
Control
Pramlintide
Control
Pramlintide
ARC
VMH
B
Number of POMC neurons (Avg/section)
80
60
40
20
0
Control
Pramlintide
C
Number of pERK-POMC neurons (Avg/section)
6
4
2
0
Control
Pramlintide
D
E
F
G
H
I
3V
100 µm
20 µm

Figure 6: Double immunohistochemical staining of leptin-induced pERK and POMC expression in the VMH and the ARC of male Sprague-Dawley rats injected at day 0 with control or LSR-pramlintide alginate beads (n=6/group), fed HF 60% and injected with leptin (IP; 5mg/kg) and perfused 45min later at day 13. Quantification of pERK in the VMH and the ARC (**A**), and of POMC (**B**) or double-positive neurons (**C**) in the ARC. Data are mean ± SEM. *P<0.05 or less using t-test. Green fluorescence labels pERK positive neurons in the control group (**D**) and in the pramlintide group (**E**). Overlayed pictures of the control group (**F**) and the pramlintide group (**G**) of pERK positive neurons labeled in green and POMC positive neurons labeled in red. Double-labeled cells appear in yellow or green surrounded with red. The white triangle indicates the position of the ARC and the white circle the position of the VMH. The yellow square indicates the area of the magnified image (40X) of double-labeled cells from the control group (**H**) and the pramlintide group (**I**). Arrows point to double-labeled neurons.

6 DISCUSSION

The goal of this study was to assess in vivo, in a larger animal model such as the rat, the long-term effect of genetically-engineered cells encapsulated in alginate beads as a new tool to deliver drugs in a controlled manner. Thus, when these encapsulated cells are stimulated via the LSR plasmid by an increase in lipids such as FFA and TG in the plasma, it will activate the transcription of the second plasmid to stimulate the transcription of pramlintide (Rossger, Charpin-El-Hamri et al. 2013). The effects of pramlintide-producing transgenic cells injected in male rats fed with a 60% high fat diet were analysed for 13 days. A decrease in cumulative body weight gain and in food intake in pramlintide releasing rats was seen transiently, i.e. in the first days post injection. No differences in daily cumulative body weight gain and daily food intake between controls and the pramlintide group were observed after this time point, even though a significant increase of plasma amylin between baseline and sacrifice was observed in the pramlintide group. We presume that this increase in measured plasma amylin levels was due to the secretion of pramlintide by the implanted cells. In addition, pramlintide production was sufficient to enhance the leptin's lowering effect on food intake and to reduce food intake during a meal test within the first week after beads implantation. Thus this study demonstrated the successful but transient effect of an autonomous genetic system providing fatty acid-induced production and secretion of pramlintide in rats put on a high fat diet.

First we assessed the performance of different combinations of the transfection of pKR135, pMG10 and pKR146, respectively, in vitro. We observed higher SEAP (which served as positive control) and pramlintide concentration in the supernatant of the fatty acid treated wells compared to the transfected wells that were not treated with linoleic acid and the non-transfected cells. Cell supernatant was analyzed for 48h period post fatty acid treatment only; thus, even though a large number of the transfected cells were still alive 48h after transfection, their ability to further produce SEAP and pramlintide was not assessed. Other studies testing the effect of synthetic gene circuits in mammalian cells were published in the last few years (Weber, Fux et al. 2002, Kemmer, Gitzinger et al. 2010, Rossger, Charpin-El Hamri et al. 2013, Rossger, Charpin-El-Hamri et al. 2013, Ye, Charpin-El Hamri et al. 2013, Rossger, Charpin-El-Hamri et al. 2014) and the in vitro effect of these synthetic gene circuits was rarely assessed for more than 4 days post transfection due to cell proliferation. The originality of our study resides in the fact that for the first time this new drug delivering system was assessed for a longer period of time in a larger animal model. Previously the in

vivo effect of these genetically engineered cells were assessed in mice between 2 and 3 days after beads implantation as a proof of principle (Rossger, Charpin-El-Hamri et al. 2013).

Amylin is known to reduce meal size through a normal meal-ending satiation process in lean and diet induced obese (DIO) mice and rats (Morley, Flood et al. 1994, Lutz, Geary et al. 1995, Roth, Hughes et al. 2006, Mack, Wilson et al. 2007). As previously demonstrated, amylin is also able to reduce body weight gain in lean and obese models (Roth, Roland et al. 2008, Turek, Trevaskis et al. 2010). Mack and al. (Mack, Wilson et al. 2007) demonstrated that a sustained amylin infusion in rats reduced body weight and food intake for up to 11 weeks. In our study, pramlintide significantly reduced the cumulative body weight gain up to 5 days post beads injection. Furthermore, cumulative body weight gain of the pramlintide group remained lower for 3 more days; even though this difference was not significant anymore, the cumulative body weight gain of rats injected with pramlintide was still reduced by 5% to 9% compared to the controls until day 8. We can therefore assume that the encapsulated cells were producing pramlintide at least for 8 days post injection. Furthermore, the significantly stronger leptin-induced anorexia observed in the pramlintide group on day 5 and 7 tends to confirm this statement. Thus, while pramlintide induced a decrease in body weight gain for the first 5 days, this was not reflected by a decrease in food intake. Indeed, the lipid-induced pramlintide release produced an inconsistent effect on food intake. Pramlintide significantly reduced daily food intake only on day 1 post injection while food intake was significantly higher on day 2. This may reflect a compensation for the decreased food intake on the previous day. These results suggest that pramlintide was sufficient to decrease body weight gain without affecting food intake, possibly by an effect on energy expenditure. Amylin has been shown to increase energy expenditure (Mack, Wilson et al. 2007, Wielinga, Alder et al. 2007, Wielinga, Lowenstein et al. 2010). Mechanisms underlying amylin's actions on energy expenditure are not yet determined and it is unclear whether these are similar to mechanisms of amylin's actions on eating (Hay, Chen et al. 2015). In addition, while previous studies have assessed the long-term effect of a constant diffusion of a pharmacological dose (50 to 100 µg/kg/day) of amylin or pramlintide (Mack, Wilson et al. 2007, Trevaskis, Coffey et al. 2008, Turek, Trevaskis et al. 2010), the present study assessed the effect of a lower dose (≈ 1µg/day) that was only released after a meal due to the increase in blood lipids (Qi, Cai et al. 2010, Rossger, Charpin-El-Hamri et al. 2013). This lower dose could thus explain the lowering of the body weight but not the food intake.

Nevertheless, the lipid-induced pramlintide release was sufficient to significantly decrease the food intake during a 4h meal test on day 3 and to enhance the anorectic effect of leptin on day 5 and 7. Indeed previous studies have demonstrated that amylin restores leptin responsiveness in leptin resistant DIO rats, which results in a synergistic effect on eating, body weight and body adiposity (Roth, Roland et al. 2008, Trevaskis, Coffey et al. 2008, Turek, Trevaskis et al. 2010). Amylin enhances the hypothalamus to respond to leptin by increasing leptin receptor and leptin signaling (Roth, Roland et al. 2008, Trevaskis, Coffey et al. 2008, Trevaskis, Lei et al. 2010, Trevaskis, Parkes et al. 2010, Turek, Trevaskis et al. 2010, Le Foll, Johnson et al. 2015). The decreased food intake during the meal test was associated with a significant increase in amylin and leptin plasma levels in the pramlintide group. Even though the control group showed a significant but smaller amylin increase as well, these rats showed no leptin increase in the plasma. While it is known that amylin is released in response to a meal (Kanatsuka, Makino et al. 1989, Young and Denaro 1998, Qi, Cai et al. 2010), the reason for the increased plasma leptin in the pramlintide group during meal test is unclear.

At sacrifice, 13 days post beads injection, no effects of the pramlintide treatment were observed. I.e., there were no significant differences regarding daily food intake, cumulative body weight gain or plasma amylin concentration at that time. Futhermore, no decrease in plasma triglycerides, HDL cholesterol or glucose was observed in the pramlintide group. It seems likely that the transfected cells were not secreting pramlintide anymore. This statement was supported by the lack of increased hypothalamic signaling in the pramlintide group. In the AP, amylin has been shown to increase pERK signaling (Potes, Boyle et al. 2012) while in the hypothalamus leptin is also able to activate pERK signaling (Bouret, Bates et al. 2012, Balland, Dam et al. 2014). Thus in this study we chose to assess pERK signaling in the ARC as a mean to assess the efficiency of pramlintide and its synergistic effect with leptin in the hypothalamus. Leptin signaling includes several pathways, including JAK-STAT signaling, that increases the transcriptional activity of POMC (Munzberg, Huo et al. 2003, Kwon, Kim et al. 2016), and ERK signaling (Banks, Davis et al. 2000). Phosphorylated STAT3 immunohistochemistry could not be performed in this study for technical reasons. Pramlintide induced no significant increase of leptin-induced pERK signaling. The stimulation of pERK in POMC positive neurons in the ARC was not different between both groups and was of approximately 6%, less than the 20% colocalization observed by Bouret et al. (Bouret, Bates et al. 2012).

Finally, to assess if the alginate beads induced inflammation (Le Foll, Johnson et al. 2015, Dunn-Meynell, Le Foll et al. 2016), plasma analysis was performed at sacrifice. The only difference between the controls and the pramlintide group at sacrifice was the significantly lower plasma Il-10 concentration in the pramlintide group. Il-10 is known to influence the inflammatory consequences of peripheral lipopolysaccharide (LPS) in the hypothalamus, attenuating LPS-induced changes in food intake and energy expenditure (Hollis, Lemus et al. 2010). However, to our knowledge, an interaction of IL-10 with amylin and/or leptin signaling in the concerned brain areas was never mentioned, thus the decrease we observed in the pramlintide group remains unclear. While it has been shown that amylin stimulates microglial IL-6 production in the VMH in order to enhance leptin-induced pSTAT3 (Le Foll, Johnson et al. 2015), to our knowledge, no study assessed if amylin or pramlintide was able to increase plasma IL-6. It is also unlikely that IL-6 brain production will diffuse into the peripheral circulation due to the blood brain barrier. The absence of an increase in IL-1β and TNF-α suggests that the intraperitoneal beads did not induce any relevant inflammation and confirm the viability of this implant in vivo to conduct longer-term studies. A caveat in this study was the absence of rats without beads implantation, however from data found in the literature (da Cunha, Ferreira et al. 2010, Holtman, van Vliet et al. 2013, Rossger, Charpin-El-Hamri et al. 2013), the pro-inflammatory cytokines levels found in this study remained relatively low and could most likely not be responsible for the decrease in body weight gain.

To further pursue the assessment of these genetically engineered cells, it would be interesting to investigate the performance of this genetic system comparing diets as well. In our study, due to the large amount of transfected cells per rat necessary for the system to show significant results, only a comparison between control rats and pramlintide rats that were all fed with high fat diet was possible. A preliminary study (data not shown) was performed using low and high fat diet groups. In that study, $4x10^6$ cells per rat were injected and while we saw an enhanced leptin effect to reduce food intake, no decrease in baseline food intake and body weight gain was observed. Thus while the technology of having pramlintide release only during a meal seems promising, this method has too many constraints and needs to be improved to be efficient in a large animal model for extended periods of time.

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